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cDNA sequence of an interactor FANCIP1 of the Fanconi anaemia protein of complementation group A

Description

Field of the invention

The present invention relates to the cDNA of an interactor FANCIP1 of the Fanconi anaemia protein of the complementation group A (FANCA) as well as the thereof coded protein. Further issues are the corresponding gene, antibodies against the protein, FANCIP1-transgenic organisms and cells as well as the use of FANCIP1 for effector screening and the pharmaceutical application of the nucleic acid, the proteins and the antibodies.

Backgroand of the invention

Fanconi anaemia (further being called FA) is an autosomal recessive inheritable disease manifested by clinical symptoms such as progressive pancytopenia, congenital malformations and higher risk of cancer (Glanz and Fraser, 1982). At least 15% of FA-patients develop myeloid leukaemia (Auerbach and Allen, 1991).

Cytogenetically FA cells are characterized by a hypersensitivity to DNA cross-linking agents, e.g. mitomycin C (MMC) and diepoxybutane (DEB), manifested by chromosomal breaks and aberrations (Auerbach, 1993). After treatment with MMC FA lymphoblasts and fibroblasts show a retardation or an arrest in the G2-phase of the cell cycle (Kubbies et al., 1985; Seyschab et al., 1995). Additionally, a higher oxygen-sensitivity of FA cells has been reported (Joenje et al., 1981; Schindler and Hoehn, 1988; Poot et al., 1996).

On the basis of somatic cell fusion studies at least eight different complementation groups (A to H) could be distinguished for FA (Joenje et al., 1997). Up to now genes for three complementation groups could be identified: FANCC (Strathdee et al., 1992; WO93/22435), FANCA (Lo Ten Foe et al., 1996; The Fanconi anaemia/Breast cancer consortium, 1996; WO98/14462) and FANCG (Saar et al., 1998; De Winter et al., 1998).

Although the molecular functioning of the FA proteins is still unknown the cellular phenotype and the higher risk of cancer through a defect gene indicate a participation in DNA repair, cell cycle regulation and/or haemotopoiesis. The similarity of the clinical and cellular phenotype of the different complementation groups and the findings that the FANCA and FANCC protein interact through FANCA phosphorylation and being transported into the cell nucleus as a complex (Kupfer et al., 1997a, Yamashita et al., 1998) point to a protein cascade or a functional co-effect in a complex. The participation in this complex could also be shown for FANCG (Garcia-Higuera et al., 1999; Waisfisz et al., 1999; Reuter et al., 2000).

Crucial progress with revealing the molecular cause for the FA pathogenesis can be obtained through the identification of the participating genes and proteins. The following FANCC interactors are published up to now: cyclin-dependent kinase cdc2 (Kupfer et al., 1997b), the chaperone GRP94 (Hoshino et al., 1998), the NADPH-cytochrome P450 reductase (Kruyt et al., 1998) and a new transcription repressor (Hoatlin et al., 1999), as FANCA interactor the nexin SNX5 (Otsuki et al., 1999), as FANCA and FANCC interactor alpha spectrin II (McMahon et al., 1999). Fanconi gene 1 and 2 have been classified as potentially relevant for the pathogenesis (Planitzer et al., 1998; WO98/16637 and WO98/45428).

It was the object of the present invention to find interactors of the Fanconi anaemia proteins FANCA and FANCC. Based upon the FA pathogenesis as a model system for mechanisms maintaining the genetic stability the goal was to identify parts of a protein complex or a protein cascade which play a role in DNA repair, cell cycle regulation and/or oncogenesis.

Summary of the invention

The present invention describes the identification of a cDNA which codes for a new protein termed FANCIP1 (Fanconi anaemia protein interacting protein 1). The cDNA sequence has been foand using an interaction trap version of the yeast two-hybrid system (Fields and Song, 1989; Finley Jr. et al., 1996) whereas the protein of the complementation group A (FANCA) has been used as bait. The protein being coded through the FANCIP1 cDNA interacts with FANCA and thus can be part of the complex or the signal

transduction cascade which leads to FA pathogenesis if defect. The FANCIP1 cDNA and the encoded protein as much as the corresponding gene and antibodies against the protein are useful as diagnostic, therapeutic or preventive tools for diseases being associated with DNA repair defects, cell cycle disorders, cytopenia, tumorigenesis and/or tumor progression. Furthermore, they can serve as targets for effector screening processes to develop new drugs for the treatment of diseases as mentioned before.

The present invention concerns one nucleic acid which contains

a) the nucleotide sequence or a protein-coding part of it shown in Fig.1
b) one of the sequence from a) within the context of the degeneration of the genetic code corresponding nucleotide sequence
c) one with the sequences from a) and/or b) ander stringent conditions hybridizing nucleotide sequence or
d) one to a sequence from a) and/or b) complementary sequence

The nucleotide sequence being shown in Fig.1 contains an open reading frame which corresponds to a protein with a length of 308 amino acids. The amino acid sequence of this protein is shown in Fig.2.

In the EST data base of the National Center for Biotechnology Information (NCBI) human cDNA clones can be foand which contain parts of the nucleotide sequence shown in Fig.1. The following human ESTs are mentioned:
Access-numbers AA165403, AA455594, AA314472, N34087, AA452340, AA182700, N41615, AA470049, AI751597, AA463289, AA132459, W31487, R56355, H58271, H16122, W77956, AA193332, AA323923, AA370209, AA296758, W72757, AA093971, AA385544, AA386175, AA165402, AW085713, H42806, AA093977, AI161152, AA370011, AI671702, R71215, AA885343, T79297, AI814869, R81567, AI082713, N29615, AW087726, AW075710, AI952608, AI818073, AI784445, AI432812, AI375568, AI372904, AI364106, AI143379, AA993074, AA953985, AA862385, AA761084, AA576229, AA569223, AA463198, AA452117, AA416877, AA074872, W16851, W04568, N40176, AW068354, AA857004, H58663, H15819, AW264944, AI923965, AI692214, AI475321, AI435987, AA961068, AA206059, AI469161, T84789, AA507257, AA707515, AA132458, AA179262, T79211, W31505, N25699, T99574, T99363, AI751598, AA713668, T91119, AW105515, AA370208, AI422128, R81568, AI038899,

AI971847, AI540650, AI826106, AA885960, R56263, AA825431, T99147, D31503 and AF049564. Among these numbers no information about a complete open reading frame or a possible biological function is given.

The search for functional domains of the FANCIP1 protein (Fig.2) using the ProfileScan Server of the ISREC Bioinformatics Group (Swiss Institute for Experimental Cancer Research) provided as the most significant result an esterase/lipase/thioesterase domain.

Besides the nucleotide sequence shown in Fig.1 and a nucleotide sequence corresponding to that sequence within the context of the degeneration of the genetic code the present invention comprises another nucleotide sequence which hybridizes with one of the sequences mentioned before. The term hybridization according to the present invention is being used as in Sambrook et al. (1989).

The nucleic acid of the present invention encloses a protein-coding part of the nucleotide sequence being shown in Fig.1 or a sequence showing a homology of more than 65% preferably more than 80% or showing a part of the sequence of preferably at least 15 nucleotids. In addition the nucleotide sequence may enclose an RNA or an analogue of the nucleic acid, e.g. a peptide-nucleic acid.

The nucleic acids of the present invention can be isolated from mammals according to known techniques using short parts of the nucleotide sequence as shown in Fig.1 as hybridization probes and/or as primer according to known methods. Nucleic acids can be furthermore produced by chemical synthesis where modified nucleotide components (e.g. methylized or 2′-O-alkylized nucleotides or phosphorthioates) can be used instead of the usual nucleotide elements. Nucleic acids consisting partly or wholly of modified nucleotide components can be used for example as a therapeutic drug such as antisense nucleic acids or ribozymes.

The present invention concerns furthermore a vector which contains at least one copy of a nucleic acid of the present invention. This vector can be any prokaryotic or eukaryotic vector containing the nucleic acid of the present invention and/or making the expression of the nucleic acid of the present invention in a suitable host cell possible. Examples for prokaryotic vectors are chromosomal vectors such as bacteriophages and

extrachromosomal vectors such as circular plasmid vectors. Examples for eukaryotic vectors are yeast vectors or vectors being suitable for higher cells such as plasmid vectors or viral vectors.

The invention also concerns a vector which preferably contains a part of at least 15 nucleotides of the sequence shown in Fig.1. Preferably this part contains a nucleotide sequence being derived from the protein-coding area of the sequence being shown in Fig.1 or from an area important for the expression of the protein. These nucleic acids are especially suitable for the production of therapeutic applicable antisense-nucleic acids.

The present invention concerns furthermore a cell being transformed with a nucleic acid or a vector, both of the present invention. The cell can as well be a prokaryotic as an eukaryotic cell. Examples for eukaryotic cells are mammalian cells in particular. Further objects are FANCIP1-transgenic organisms such as knock-in or knock-out animal models. Animal models stably expressing the product of the nucleic acid are being called knock-in animal models, those whose corresponding gene has been destroyed are being called knock-out animal models.

The present invention includes a protein coded by a sequence as mentioned above. This protein contains the amino acid sequence as shown in Fig.2 or a homology of more than 60% preferably more than 70% to the amino acid sequence shown in Fig.2. The invention also concerns variations and fragments of the protein being shown in Fig.2. Variations are sequences which differ from the amino acid sequence shown in Fig.2 by substitution, deletion and/or insertion of individual amino acids or short amino acid chains. Among these are naturally existing allelic variations or splicevariations of FANCIP1 as well as proteins produced by means of recombinant DNA technology, especially proteins obtained through in vitro-mutagenesis using chemically synthesized oligonucleotides which regarding their biological and/or immunological activity mostly respond to the protein shown in Fig.2. This definition also includes chemically modified polypeptides. Among these are polypeptides having been modified at the termini and/or at reactive amino acid side groups through acylation or amidation.

The invention also concerns procedures leading to the production of the protein of the present invention including the cultivation of transformed cells as much as the isolation of the protein of the present invention.

Furthermore, the invention concerns the use of the polypeptide of the present invention or fragments of this polypeptide as immunogen for the production of antibodies. The production of antibodies can take place by usual means of immunizing experimental animals with the complete polypetide or fragments thereof followed by obtaining the resultant polyclonal antiserum. Monoclonal antibodies can be produced using known methods. The present invention covers antibodies against FANCIP1 or a variation of it, too.

FANCIP1 encoded by the nucleic acid of the present invention can be used as a target for a specific search for effectors. Substances having an inhibitory or activating effect on the protein of the present invention are able to influence selectively the cell functions being usually regulated by the protein itself. Therefore they can be used for the therapy of appropriate clinical pictures, e.g. cytopenia or tumors. A part of the invention is also a method for identification of effectors of FANCIP1 where cells expressing the protein are being brought into contact with different potential effector substances and the cells are being analysed in regard of changes, e.g. cell activating, cell inhibiting, cell proliferation and/or cell genetic changes. By this means binding domains of FANCIP1 can be identified. Part of the invention are pharmaceutically effective effector-substances which are gained by the method described above.

The present invention also concerns a pharmaceutical composition containing nucleic acids, vectors, cells, polypetides, antibodies and/or effector-substances as described earlier as active components and also may carry usual pharmaceutical carrier, auxiliary and/or additive substances as much as other active components. The pharmaceutical composition can be used specifically for diagnosis, therapy or prevention of diseases being associated with DNA repair defects, cell cycle disorders, cytopenia, tumorigenesis and/or tumor progression. This is also valid for the diagnosis of a predisposition for such diseases in individuals especially for the diagnosis of a risk of cytopenia and/or tumor diseases. Furthermore a focused diagnosis of diseases being connected with direct or indirect changes of the activity of FANCIP1 is made possible. Using specific nucleic acid probes

these examinations can be accomplished at the nucleic acid level, e.g. at gene or transcription level, or with the help of antibodies against FANCIP1 at the protein level.

With clinical pictures being traced back to a breakdown of FANCIP1 a gene therapeutical treatment can follow which includes the transmission of a nucleic acid encoding FANCIP1 via vectors, e.g. viral vectors, into the corresponding final tissue. On the other side a genetherapeutical treatment can take place on clinical pictures tracing back to an uncontrolled expression of FANCIP1 which leads to the blockade of this expression.

The present invention also includes a method for the diagnosis of the diseases mentioned above where contact between a patient or a sample from the patient, e.g. a sample of a body liquid or of a tissue, and a pharmaceutical composition of the invention is established and where the nucleotide sequence and/or the expression of the nucleic acid of the invention is determined qualitatively or quantitatively. These methods of determination can take place at the level of nucleic acids by using nucleic acid hybridization probes or through reverse transcription/PCR and at the protein level by using antibodies in cyto- or histochemical methods respectively. The pharmaceutical composition can be used as a marker for the appearance of cytopenias, tumors or other diseases being connected with proliferation or a predisposition for the named pathophysiological changes.

Finally, the present invention includes a procedure for a therapy or prevention of one of the diseases mentioned above where the patient is given a pharmaceutical composition of the present invention including the active component in an effective amount for the disease. Specific examples for pharmaceutical compositions being suitable for therapeutic use are amongst others bispecific antibodies and antibody-toxins and antibody-enzyme conjugates respectively. Other favoured pharmaceutical compositions for therapeutical use are antisense nucleic acids, gene therapy vectors or effector substances, e.g. in form of low molecular activators or inhibitors.

Detailed description of the invention

Interaction trap

For the cloning of cDNAs whose gene products interact with the Fanconi anaemia protein FANCA and therefore may play a role in the FA pathogenesis an interaction trap version of the yeast two-hybrid system has been used.

For the construction of the FANCA bait protein the complete coding sequence of the FANCA protein has been cloned into the vector pEG202 by using the EcoRI site within the reading frame with the region encoding the LexA DNA-binding domain. For the expression of the prey protein the vector pJG4-5 has been used allowing the construction of fusion proteins with the B42-transactivation domain. Using the FANCA bait protein a HeLa cDNA library being cloned into this vector as a fusion gene bank has been screened.

The yeast strain EGY48 has been used as the host organism. Proof of a positve interaction was given through transcriptional activation of the LEU2 gene from which the growth of yeast on leucine-free medium results.

Before implementation of the interaction trap it has been guaranteed that no intrinsic trans-activating characteristics of the FANCA-bait-fusion construct exists by spreading pEG202FANCA transformed EGY48 yeasts on glucose medium without histidine and leucine.

With pEG202FANCA and the B42-fusion-cDNA-bank co-transformed EGY48 have been preselected based on the existence of both vectors on leucine-containing medium and have been taken up. For the search of interacting yeast clones aliquots have been spread on leucine-free medium and incubated 3 to 5 days at 30°C. Altogether aliquots according to an amount of $1x10^{6}$ transfectants have been screened. The dependence of the transcriptional activation of positive clones upon the expression of the prey protein has been tested on leucine-free medium. The isolation of the interactor plasmids has been carried out by growing yeasts in glucose-medium without tryptophane, electroporation of the nucleic acid isolate in the E.coli strain XL1blue (Stratagene) and plasmid preparation of bacteria cells. For confirmation of the interactions retransformations of the isolated prey interactor, in combination with different bait structures, have been carried out. The observed interaction has been verified in combination with pEG202FANCA. In addition possible interactions with the LexA fusion partner could be excluded by co-retransformation with the pEG202 empty vector on the one hand and with a LexA-DNA-ligase-bait fusion construct as a negative control on the other hand.

Sequence analysis of the FANCIP1 cDNA

The length of the gene bank cDNAs of the isolated interactor clones has been determined through EcoRI/Xhol restriction hydrolysis. The initial sequencing of the cDNAs took place through an automated cycle sequencing method (Applied Biosystems) using the nucleic acid primer Bco I (5′- ACC AGC CTC TTG CTG AGT GGA GAT G-3′). The complete sequencing of the vector with inserted FANCIP1 cDNA fragment occurred with the nucleic acid primers BcoI and BcoII (5′- GAC AAG CCG ACA ACC TTG ATT GGA G-3′) done by the company Sequence Laboratories Göttingen.

For determination of the 5′ part sequence of the foand nucleotide sequence the 5′/ 3′RACE Kit (Boehringer Roche) has been used. The following sequence specific primers have been used: FANCIP1-SP1 (5′-GGG GGC AGG AAT ATG AGA GG-3′) and FANCIP1-SP2 (5′-TTT AGG GGG AAG TGT ACC TG-3′). The received PCR product has been cleaned electrophoretically (JETquick Gel Extraction Kit, GENOMED) and directly sequenced using the T7 Sequenase Version 2.0 DNA Sequencing Kit (Amersham-Pharmacia) and the primer FANCIP1-SP2 as named above. The belonging of the obtained nucleotide fragment to the plasmid-inserted interactor fragment has been verified through an overlapping sequence area of 38 nucleotides. The assembled nucleotide sequence delivered a cDNA area being 1553 nucleotides long including a part of the 5′ untranslated region, the whole open reading frame of 924 nucleotides and 308 codons respectively and the almost complete 3′ untranslated region up to the polyadenylation signal (AATAAA).

In order to find similar nucleotide sequences in the sequence data base of the National Center of Biotechnology Information (NCBI) the cDNA sequence of FANCIP1 (Fig. 1) has been analysed using the Blast program at the NCBI (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=1). Significant homologies to human clones only occurred in the EST database but neither included a complete open reading frame nor information to a possible biological function.

For the determination of potentially functional domains within the FANCIP1 protein the amino acid sequence (Fig. 2) has been analysed using the ProfileScan server of the ISREC Bioinformatics Group (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html).

Short description of the figures

Fig.1 (SEQ ID NO.1) a nucleotide sequence including the open reading frame encoding FANCIP1,

Fig.2 (SEQ ID NO.2) the amino acid sequence of an open reading frame of the nucleotide sequence shown in Figure 1,

Fig.3 (SEQ ID NOs. 3 and 4) the nucleic acid primer used for the sequencing of the plasmid-inserted FANCIP1 nucleotide sequence,

Fig.4 (SEQ ID NOs. 5 and 6) the nucleic acid primer used for the 5′ RACE analysis.

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